Mechanistic Insight into Pertussis Toxin and Lectin Signaling Using T Cells Engineered To Express a CD8α/CD3ζ Chimeric Receptor

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ABSTRACT: Mammalian cell-surface receptors typically display N- or O-linked glycans added post-translationally. Plant lectins such as phytohemagglutinin (PHA) can activate the T cell receptor (TCR) and other cell-surface receptors by binding to glycans and initiating receptor cross-linking. Pathogenic microorganisms such as Bordetella pertussis also express proteins with lectin-like activities. Similar to plant lectins, pertussis toxin (PTx) can activate the TCR and bind to a variety of glycans. However, whether the lectin-like activity of PTx is responsible for its ability to activate TCR signaling has not been formally proven. Here we examined the ability of PTx and a panel of lectins to activate the TCR or a CD8α/CD3ζ chimeric receptor (termed CD8ζ). We demonstrate that CD8ζ rescues PTx-induced signaling events lacking in TCR null cells. This result indicates that CD8ζ can substitute for TCR and supports the hypothesis that PTxB (functioning as a lectin) stimulates signaling via receptor cross-linking rather than by binding to a specific epitope on the TCR. Moreover, PTx is able to activate signaling by binding either N-linked or O-linked glycan-modified receptors as the TCR displays N-linked glycans while CD8ζ displays O-linked glycans. Finally, studies with a diverse panel of lectins indicate that the signaling activity of the lectins does not always correlate with the biochemical reports of ligand preferences. Comparison of lectin signaling through TCR or CD8ζ allows us to better define the structural and functional properties of lectin–glycan interactions using a biologically based signaling readout.
structure. The S1 subunit of PTx, S1, is an ADP-ribosyltransferase that targets the α-subunit of some GTP-binding proteins.10 The five B subunits of PTx (collectively termed the B pentamer or PTxB) are required for binding and cytosolic entry of S1 into mammalian cells. Unlike other AB5 toxins, which have five identical B subunits, PTxB is comprised of four different subunits (S2−S5) in a 1:1:2:1 ratio. All the binding activities have thus far been mapped to the S2 and S3 subunits of PTxB.11−22 Analogous to WGA, each S2 and S3 subunit contains multiple glycan binding sites. Interestingly, although the sequences of S2 and S3 are 71% identical, each has distinct binding preferences. PTx has been shown to bind a broad array of glycans, including sialylated and nonsialylated N-glycans, sialylated O-glycans, and sialylated gangliosides.23

The glycan binding activity of PTxB mediates activities independent of its role in delivering the S1 catalytic subunit to cellular targets. Via the B subunits, PTx can bind to a variety of cellular receptors and activate their associated signaling pathways.24 Receptor targets for PTxB include the TCR in T cells, Toll-like receptor 4 (TLR4) in dendritic cells, and CD14 in myelomonocytic cells.25−33 In T cells, the binding of PTxB to the TCR leads to T cell activation and mitogenesis.34 The various receptors that PTxB bind share little structural similarity but are heavily glycosylated.35−36 The broad binding specificity of PTx likely allows it to act as a superlectin, able to trigger signaling by clustering a wide variety of glycosylated receptors.37−42 This hypothesis, however, has yet to be formally proven.

Studies that aim to improve our understanding of the molecular basis of lectin activity in cellular systems have been hampered by several factors. One problem is the incomplete understanding of the repertoire of binding sites for each particular lectin. Glycans also typically interact with their cognate binding sites with very low affinity, and tight binding is often achieved by engaging multiple binding sites. Additionally, because cell-surface glycans are built by sequential enzymatic processing, they can be in various stages of "completion," resulting in considerable heterogeneity. Furthermore, a single cell-surface protein may display both N-linked and O-linked glycosylation. Some of these problems have been overcome by the development of glycan arrays similar to that established by the Consortium for Functional Glycomics. These arrays can be very useful for identifying binding sites for lectins by identifying similar motifs within different glycans. However, technical issues can complicate the analysis, necessitating additional confirmatory studies. For example, if the glycans on the array are not spaced to allow for engagement of multiple binding sites or if the lectin in question binds multiple different ligands by distinct mechanisms, array-type experiments are less useful. A cellular system in which some of these variables could be experimentally controlled would contribute valuable information to our understanding of PTxB and lectin action.

Figure 1. Schematic representation of TCR and CD8ζ structure, glycosylation patterns, and signaling ability. (A) Schematic representation of the TCR complex showing the α, β, γ, δ, ε, and ζ chains. Disulfide bonds are represented by dashed lines; immunoglobulin-like domains are represented by three-quarter circles, and cytoplasmic ITAM motifs are represented by white ovals. N-Linked glycans representative of those typically found on the TCR are shown in CFG symbol nomenclature (key in panel D). (B) Schematic representation of the CD8ζ chimeric protein (315 amino acids). Residues 1−203 are from CD8α and contain an Ig-like fold, a stalklike region, and a transmembrane domain. Residues 204−315 are from the cytoplasmic domain of CD3ζ, which contains three ITAM motifs. The chimera is expressed as a disulfide-linked homodimer, with extensive O-linked glycosylation in the stalklike region. (C) Schematic representation of the signaling pathway downstream of ζ chain phosphorylation that can be activated by clustering either TCR α/β-chains or CD8ζ chimeric molecules. (D) Representative examples of N-linked and O-linked glycans typically found on human T cells according to the CFG glycan profiling database. Glycans are shown in CFG symbol nomenclature. N represents asparagine and S/T serine/threonine. n indicates the number of modified branches.
PTxB activates a canonical TCR/CD3 signaling pathway, including events such as phosphatidylinositol (PI) hydrolysis, Ca\(^{2+}\) flux, and MAP kinase activity.\textsuperscript{24,43,44} Furthermore, while wild-type Jurkat cells robustly respond to PTxB, Jurkat derivatives lacking the TCR are devoid of any detectable TCR-related signaling responses to PTxB.\textsuperscript{24} The lack of PTx-induced signaling in cells missing the TCR provides the opportunity to engineer TCR minus mutant cell lines to express exogenously added “receptor” components to rescue PTx-induced signaling. In this report, we used the system previously described by Irving et al. to engineer a TCR minus Jurkat T cell line to express a chimeric protein containing the extracellular CD8\(\zeta\) domain and the intracellular CD3\(\zeta\) domain (CD8\(\zeta\) chimera).\textsuperscript{45,46} Comparing the responses of the CD8\(\zeta\) chimera to those of the wild-type TCR is a useful model system for studying the mechanism of action of molecules such as PTx, ConA, PHA, etc. Additionally, this model system can provide important insights into the structural and functional properties of TCR activation.

### MATERIALS AND METHODS

**Cell Lines and Reagents.** The human Jurkat T cell lymphoma line (clone E6.1) was obtained from the ATCC, while the J.EMS-T3.3\textsuperscript{47,48} Jurkat derivative lacking TCR expression was obtained from A. Weiss (University of California, San Francisco, CA). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). PTx and PTxB were purchased from List Biologicals. The presence of contaminating PTx holotoxin in PTxB preparations was assessed using the CHO cell clustering assay.\textsuperscript{49} The concentration of residual PTx holotoxin in PTxB preparations was found to be >2%. No effect was observed in experiments using the PTx holotoxin at these concentrations in short-term assays; therefore, we conclude that the observed effects are due to PTxB and not contaminating catalytic subunit activity. PTx was inactivated by being boiled for 30 min. Stimulating antibodies, αCD8 monoclonal antibody (mAb) clone OKT8 and αCD3 mAb clone HIT3a, were purchased from eBioscience and BD Pharmingen, respectively. Antibodies used in immunoprecipitation assays, anti-ZAP-70 (clone 2F3.2) and the anti-phosphotyrosine antibody (clone 4G10), were purchased from Upstate, and random sequence IgG antibodies were purchased from Covance. Protein concentrations were determined using the Bio-Rad protein assay with BSA as standard. For all experiments, antibodies were used at a concentration of 10 μg/mL.

**Table 1. Signaling and Binding Activity of Bacterial and Plant Lectins**

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Abbreviation</th>
<th>Preferred Ligand</th>
<th>Protein glycan targets</th>
<th>Ca(^{2+}) signaling</th>
<th>ERK signaling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrina cristagalli lectin</td>
<td>ECL</td>
<td>Galβ1−4GlcNAc</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Jacalin (Artocarpus integrifolia)</td>
<td>JAC</td>
<td>Galβ1−3GalNAc (T antigen)</td>
<td>O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vicia villosa agglutinin</td>
<td>VVA</td>
<td>GalNAc1-Ser/Thr (Tn antigen)</td>
<td>O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phaseolus vulgaris agglutinin</td>
<td>PHA-E</td>
<td>GlcNAc1−2Man1−3(GlcNAc)−4 (Galβ1−4GlcNAc)−1−2Man1−6Man</td>
<td>N-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bordatella pertussis toxin</td>
<td>PTx</td>
<td>NeuAc, complex-type N-glycans</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>wheat germ agglutinin</td>
<td>WGA</td>
<td>GlcNAc, NeuAc</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>succinylated wheat germ agglutinin (T. aestivum)</td>
<td>sWGA</td>
<td>GlcNAc</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>soybean agglutinin (Glycine max)</td>
<td>SBA</td>
<td>terminal GalNAc</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ricinus communis agglutinin</td>
<td>RCA</td>
<td>terminal Gal</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>P. vulgaris leucoagglutinin</td>
<td>PHA-L</td>
<td>Galβ1−4GlcNAc−1−2Man</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Datura stramonium lectin</td>
<td>DSL</td>
<td>GlcNAc−1−4, Galβ1−4GlcNAc</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lens culinaris agglutinin</td>
<td>LCA</td>
<td>branched (Man2) with Fucα1−6GlcNAc</td>
<td>N-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pisum sativum agglutinin</td>
<td>PSA</td>
<td>branched (Man2) with Fucα1−6GlcNAc</td>
<td>N-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Concanavalin A (Canavalia ensiformis)</td>
<td>ConA</td>
<td>branched (Man3),</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Griffonia simplicifolia lectin II</td>
<td>GSI II</td>
<td>terminal GlcNAc</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Lycopersicon esculentum lectin</td>
<td>LEL</td>
<td>GlcNAc</td>
<td>N-glycans, O-glycans</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Dolichos biflorus agglutinin</td>
<td>DBA</td>
<td>GalNAc1−3GalNAc, GalNAc1−3(Fucα1−2)Gal (A antigen)</td>
<td>N-glycans, O-glycans</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>peanut agglutinin (Arachis hypogaea)</td>
<td>PNA</td>
<td>terminal Galβ1−3GalNAc (T antigen)</td>
<td>O-glycans</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sophora japonica agglutinin</td>
<td>SJA</td>
<td>terminal GalNAc/Gal</td>
<td>N-glycans, O-glycans</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ulex europaeus agglutinin</td>
<td>UEA-I</td>
<td>Fucα1−2Gal (H antigen)</td>
<td>N-glycans, O-glycans</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*The stated ligand preferences are based on information available from public databases [Consortium for Functional Glycomics (http://www.functionalglycomics.org) and Lectin frontier Database (http://riodb.ibase.aist.go.jp/rcmg/glycodb/LectinSearch)] and published reports of lectin–glycan binding.\textsuperscript{1,9,23,62–64} ND means not determined.*
were obtained from Upstate Biotechnology. Western blotting antibodies for phosphorylated and total p44/42 ERK MAP kinases were obtained from Cell Signaling Technologies. The CD8ε chimera construct (comprised of the extracellular and transmembrane domains of CD8α fused to the cytoplasmic domain of CD3ε) was a kind gift from A. Weiss.

Construction of a Jurkat Derivative Cell Line Expressing the CD8ε Chimera. The CD8ε chimera was subcloned into the Hpal site of the Mig-R1 retroviral vector (Mig-R1/CD8ε). Mig-R1 is a bicistronic retroviral vector containing a multiple-cloning site upstream of an internal ribosome entry site followed by the cDNA encoding enhanced green fluorescent protein (GFP).32 The Mig-R1 and Mig-R1/CD8ε constructs were cotransfected with the envelope construct encoding pantropic VSVg into GP-293 cells (HEK cells stably expressing retroviral gag and pol) (Clontech). Jurkat cells were then transduced with the HEK supernatant by spinfection at 1800 rpm for 45 min, and gene expression from cells were then transduced with the HEK supernatant by spinfection at 1800 rpm for 45 min, and gene expression from cells stably expressing retroviral construct encoding pantropic VSVg into GP-293 cells (HEK cells stably expressing retroviral gag and pol) (Clontech). Jurkat cells were then transduced with the HEK supernatant by spinfection at 1800 rpm for 45 min, and gene expression from Mig-R1 was monitored by FACS analysis for GFP. After several weeks in culture, the top 10% of GFP-expressing cells from each population were selected using a FACSARia cell sorter and expanded. Expression of the CD8ε chimera was analyzed by flow cytometric analyses of live cells stained with the anti-CD8α mAb (OKT8) primary antibody and anti-mouse PE secondary antibody.

Immunoprecipitations and Western Blotting Assays. Jurkat cells (2.5 × 10⁶) were washed once with PBS and suspended in serum-free RPMI. The samples were slowly warmed to 37 °C and then stimulated with 10 μg/mL anti-CD3 mAb, anti-CD8 mAb, or PTxB for 5 min. Samples were washed once with ice-cold PBS and lysed in lysis buffer [1% NP-40, 0.1% SDS, 0.1% deoxycholate, 150 mM NaCl, 50 mM Tris, 20 mM Na₃VO₄, and Complete protease inhibitor tablets (Roche)]. Lysates were clarified by centrifugation, and supernatants containing 1 μg of immunoprecipitating antibody per 100 μg of total protein were incubated overnight at 4 °C with 30 μL of 60% protein A/G bead slurry (Calbiochem) or combined with 3× Laemmli sample buffer for whole cell extracts. Beads were washed four times with 1 mL of lysis buffer, suspended in 3× Laemmli sample buffer, boiled for 10 min, and electrophoresed in 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels.24 The gels were transferred to nitrocellulose, probed with the appropriate primary and secondary antibodies, and visualized via chemiluminescence (Super Signal, Pierce).

Measurement of ERK Activity. Cells (1 × 10⁶ per treatment) were serum starved when indicated and stimulated for various times with 2 or 5 μg/mL PTxB, 2 μg/mL α-CD3, or 2 μg/mL anti-CD8 mAb. After the indicated treatment, cells were lysed directly in Laemmli sample buffer and analyzed for phosphorylated ERK1 and 2 (phospho-ERK) and total ERK by Western blotting.24

Inositol Phosphate Assays. Jurkat cells (5 × 10⁶ cells/mL) were labeled with 2 μCi/mL [3H]myo-inositol (Perkin-Elmer) for 18 h in RPMI containing 10% serum. The cells were washed with cold PBS and suspended at a density of 10⁶ cells/mL in serum-free RPMI containing 20 mM LiCl; 10⁶ cells/treatment were stimulated with designated amounts of 5 or 10 μg/mL PTxB, 2 μg/mL anti-CD3 mAb, 2 μg/mL anti-CD8 mAb, or 10 μg/mL PHA for 2 h at 37 °C. After stimulation, the cells were lysed with 0.4 M perchloric acid at 4 °C for 15 min and neutralized with 0.72 M KOH and 0.6 M KHCO₃. Total inositol phosphates were isolated with Dowex resin (Bio-Rad), washed with water, and eluted with 1 M ammonium formate and 0.1 M formic acid. The eluted samples were counted with a liquid scintillation counter, and the percent conversion of inositol phosphates from total incorporated ³H was calculated.24 Data were graphed and analyzed using GraphPad Prism 4.

Ca²⁺ Flux Assays. Cells (2 × 10⁵ cells/sample) were washed with serum-free RPMI and plated. Cells were loaded with the Fluo-4 NW Calcium Assay Kit (Invitrogen) according to the manufacturer’s protocol and treated with PBS (without Ca²⁺ or Mg²⁺), PTxB, αCD8 mAb, αCD3 mAb, or lectin (2 μg/mL). Ca²⁺ flux was analyzed by the increase in fluorescence reported by the Flex Station II (Molecular Probes) using SoftMax Pro. Data were imported into GraphPad Prism 4 for analysis and production of graphs.

Aggregation Assays. Cells were washed with serum-free HBSS (Gibco) and plated in 96-well microtiter plates at a density of 1 × 10⁶/mL. Cells were treated with HBSS, PTxB, or lectin (2 μg/mL). The microtiter plates were then incubated at 37 °C and observed for agglutination after 1–2 h.

RESULTS

Generation and Characterization of TCR Minus Jurkat Cells Stably Expressing the CD8ε Chimera. To gain mechanistic insight into bacterial and plant lectin signaling through Ig superfamily receptors such as TCR/CD3, we sought to investigate lectin-mediated activation of a chimeric “receptor” termed CD8ε, which contains the extracellular and transmembrane portions of human CD8α chain fused to the intracellular cytoplasmic domain of CD3ζ (Figure 1B).45,46,53,54 Previous studies indicated that this chimera activates a classical signaling pathway similar to that activated by TCR/CD3 (Figure 1C).45,46 Additional studies with the CD8ε chimera demonstrated that signaling through CD3 can be propagated by clustering or aggregating the three cytoplasmic ITAM motifs found in each CD3ζ molecule, and that signaling through CD3 does not necessarily require ligand-induced conformational changes in the α/β-chains of the TCR.35,46,55,56 Interestingly, the extracellular ligand binding domains of the TCR and CD8α differ in their glycosylation status; the TCR α/β-chains contain predominantly N-linked glycans, while CD8α contains only O-linked glycans.35,53,57 Computational profiling of the TCR and CD8 polypeptides supports these data as the TCR α-chain contains eight predicted sites of N-linked glycosylation while the CD8 α-chain contains seven predicted sites of O-linked glycosylation (Table S1 of the Supporting Information).58,59 By comparing PTx- and lectin-stimulated signaling through the TCR or the CD8ε chimera, we can ask specific questions regarding the potential role of the peptide backbone structure and glycosylation status in receiving and propagating the PTx/lectin signal.

The CD8ε chimeric gene was transduced into the TCR negative Jurkat T cell line (clone J.EMS-T3.347,48) using a Mig-R1/CD8ε retrovirus. Because Mig-R1/CD8ε drives gene expression with a bicistronic mRNA simultaneously expressing GFP and CD8ε,24 we were able to use cell sorting based on high-level GFP expression to generate a stable CD8ε positive cell line. Cells transduced with the empty Mig-R1 retrovirus vector (designated TCR−) expressed high levels of GFP (Figure 2A) compared to untransduced controls (data not shown) but did not express any detectable CD8 or CD8ε (Figure 2B). In contrast, cells transduced with the Mig-R1/CD8ε retroviral vector (designated CD8ε) expressed high
levels of GFP (Figure 2C) and also expressed high levels of CD8ζ (Figure 2D). These cells, which express CD8ζ on a TCR null background, serve as the basis for the following studies.

CD8ζ Substitutes for the TCR and Rescues PTx Signaling in TCR− Jurkat Cells. Next, we determined if expression of the CD8ζ chimera could rescue PTxB-stimulated signaling events, which occur in WT Jurkat but not in TCR minus Jurkat cells.24 WT, TCR−, or CD8ζ cells were stimulated for 2 h with PTxB, anti-CD3, or anti-CD8 antibodies and analyzed for the production of inositol phosphates (InsP) (Figure 3). Consistent with previous reports,24,43,45 WT Jurkat cells exhibited increased InsP levels when treated with the anti-CD3 antibody (Figure 3A), CD8ζ cells exhibited increased InsP levels when treated with the anti-CD8 antibody (Figure 3C), and TCR− cells exhibited no detectable response to either antibody (Figure 3B). PTxB strongly induced the accumulation of InsP in WT cells (Figure 3A), but not in TCR− cells (Figure 3B), and expression of CD8ζ rescued the ability of PTxB to induce InsP accumulation (Figure 3C). This result indicates that CD8ζ can substitute for the TCR and supports the hypothesis that PTxB (functioning as a lectin) stimulates signaling via receptor cross-linking and/or clustering rather than by binding to a specific epitope in the TCR α/β-chains and initiating signaling via a conformational change in the α/β-chains themselves.

PTxB-Mediated Signaling through the CD8ζ Chimera Involves Canonical TCR Signaling Components. On the basis of the observation that PTxB signaling was rescued by expression of the CD8ζ chimera, we next determined whether PTxB-activated CD8ζ signaling events were similar to those typically initiated by the TCR.45,46,55 WT Jurkat cells stimulated with PTxB or anti-CD3 antibodies exhibited increased levels of tyrosine phosphorylation of proteins at molecular masses of 100, 70, and 35 kDa, consistent with that reported for canonical TCR signaling and observed in earlier studies by our laboratory (Figure 4A).24 Interestingly, CD8ζ cells stimulated with PTxB or anti-CD8 antibodies demonstrated increases in tyrosine phosphorylation similar to that observed in WT cells stimulated with PTxB or anti-CD3 antibodies, indicating that the CD8ζ chimera activates a signaling pathway very similar to that of TCR/CD3.45,46,55,60−62 Increases in the level of tyrosine phosphorylation were not seen in TCR− Jurkat cells following treatment with PTxB, anti-CD3, or anti-CD8 antibodies (data not shown).24

Phosphorylation of ZAP-70 is thought to be the most critical event in TCR activation, as it is responsible for the phosphorylation of downstream proteins, including SLP-76 and PLCγ1.50,60,62 To determine if ZAP-70 was tyrosine-phosphorylated and therefore active in CD8ζ cells stimulated with PTxB, we stimulated WT or CD8ζ Jurkat cells with PTxB or the appropriate control antibody, isolated ZAP-70 by immunoprecipitation, and probed Western blots with anti-phosphotyrosine or anti-ZAP-70 antibodies. WT Jurkat cells treated with an anti-CD3 antibody or PTxB exhibited increased levels of tyrosine phosphorylation of ZAP-70 (Figure 4B). Similarly, CD8ζ cells treated with the anti-CD3 antibody or PTxB displayed increased levels of tyrosine phosphorylation of ZAP-70 (Figure 4B).
PTxB-induced InsP accumulation was a result of CD8ζ signaling and not low-level TCR expression (Figure 5C). Thus, as in InsP accumulation experiments described above, PTxB can also activate the ERK MAP kinase pathway in T cells using either the TCR or CD8ζ.

**PTx Utilizes CD8ζ To Induce Calcium Signaling.** In addition to accumulation of InsP and MAP kinase activity, PTxB has been shown to promote a rapid and prolonged elevation of the intracellular level of Ca²⁺ in T cells in a TCR-dependent manner and therefore provides a platform for confirming the effects of PTxB signaling through the CD8ζ chimera.²⁴,³¹ Ca²⁺ flux in response to anti-CD3, anti-CD8, or PTxB was examined in all three Jurkat cell derivatives (Figure 6). The anti-CD3 antibody stimulated Ca²⁺ flux only in WT cells (Figure 6A), and the anti-CD8 antibody stimulated Ca²⁺ in only the CD8ζ-expressing cells (Figure 6B). WT and CD8ζ-expressing cells stimulated with PTxB displayed a rapid and sustained calcium response that remains elevated for more than 5 min post-PTxB addition (Figure 6C), although the magnitude of the PTxB response was smaller than that observed for anti-CD3 or anti-CD8. The TCRζ cells exhibited a transient spike in the intracellular level of Ca²⁺ in response to PTxB (Figure 6C). This response was nonspecific and unrelated to the biological activity of the toxin itself, as cells stimulated with 5 μg/mL boiled PTxB (Figure 6D) or 5 μg/mL BSA (data not shown) also demonstrated this rapid transient spike. Altogether, these Ca²⁺ results support our earlier studies indicating that PTxB can utilize either the TCR or the CD8ζ chimera to induce rapid signaling in T cells.

**Comparison of the Ability of Plant Lectins To Signal through the TCR or CD8ζ.** Many of the plant lectins are known to activate TCR signaling via the binding of glycan moieties typically found on the TCR α- and β-chains.⁶³ However, it remains unknown if these lectins would function like PTxB and exhibit biological activity toward other Ig superfamily members or, more specifically, would be capable of activating the CD8ζ chimera. Therefore, we compared the ability of various lectins to activate calcium signaling through the TCR (in WT Jurkat cells) or the CD8ζ chimera (in the CD8ζ stables). Both wheat germ agglutinin (WGA) and soy bean agglutinin (SBA) function like PTxB in that they stimulated Ca²⁺ signaling via either TCR or CD8ζ (Figure 7A,B). This is consistent with their ability to bind either N-linked (TCR) or
O-linked (CD8ζ) glycans (Table 1). Phytomagglutinin leucoagglutinin (PHA-L) and concanavalin A (ConA) are also capable of activating Ca2+ signaling via the TCR, but not via CD8ζ (Figure 7C,D), which is consistent with their ability to primarily bind N-linked glycans (Table 1). In contrast, peanut agglutinin (PNA) and Dolichos biflorus agglutinin (DBA) are unable to activate either receptor type (Figure 7E,F).

To validate our assay using the CD8ζ chimera as a biological tool to explore aspects of lectin binding and signaling, we examined PHA-L-mediated accumulation of InsP. PHA-L is able to induce Ca2+ signaling through the TCR, but not through CD8ζ (Figure 7C), and therefore, we would expect PHA-L to behave similarly in InsP assays. As predicted, PHA-L is indeed capable of inducing the accumulation of InsP in WT Jurkat cells but is unable to stimulate the accumulation of InsP in CD8ζ cells (Figure 8). Because PHA-L is known to bind predominantly to complex N-glycans on the TCR63 (Table 1 and Figure 1) but not to O-glycans on CD8, our results demonstrate that comparing lectin signaling via the TCR versus CD8ζ is a useful approach to exploring lectin–glycan interactions on a biological level. Specifically, we can differentiate between lectin responses mediated by receptors decorated with N- or O-linked glycans.

Next, we broadened our study to include a large panel of plant lectins (Table 1) that were selected to represent a diverse cross section of lectins that have the ability to bind to an equally diverse set of ligands. The preferred binding ligand denoted for each lectin in Table 1 is based on a consensus of published reports and glycoarray results, although from analyses of glycoarray results, it is clear that lectin–glycan binding cannot be simplified to state that a particular lectin can interact with only one particular ligand (Table S2 of the Supporting Information). The abilities of these lectins to activate downstream signaling events in WT, CD8ζ, or TCR− cells were assessed by monitoring Ca2+ responses (Table 1). The signaling responses for many lectins (WGA, sWGA, SBA, RCA, LCA, PSA, and ConA) were entirely consistent with their predicted abilities to bind N-linked or O-linked glycans. We also examined ERK activation, and the results are largely consistent with the observed Ca2+ responses (Table 1). However, some lectins exhibited biological responses not predicted by their binding preferences (Table 1 and Figure 9). For example, while ECLJ, JAC, and VVA require either the TCR or CD8ζ to induce Ca2+ signaling (Figure 9A–C), these lectins were able to activate ERK signaling in cells lacking the TCR or CD8ζ (Figure 9D,E). This suggests that these lectins activate a receptor(s) linked to ERK, but distinct from one that uses the canonical TCR signaling pathway involving ZAP-70, PLCγ1, etc.

Several lectins (DBA, PNA, SJα, and UEA-1) did not display any signaling activity in our assays, suggesting that neither TCR nor CD8ζ displays the appropriate glycans for these lectins, or they were unable to cross-link the receptors in a way that promotes activation. Because lectin activity is commonly associated with the ability to aggregate cells, each of the three cell types (WT, CD8ζ, and TCR−) was incubated with the lectins in Table 1, and cellular aggregation was assessed (data not shown). Lectin-mediated cellular aggregation was seen in all cases where cellular signaling (Ca2+ and/or ERK activation) was observed. In contrast, cells treated with DBA, PNA, SJα, and UEA-1 (which failed to induce Ca2+ or ERK signaling) did not display any aggregation, suggesting that Jurkat cells fail to display the terminal glycan required for responsiveness to these lectins.

### DISCUSSION

A CD8/CD3ζ chimeric receptor engineered to artificially link the extracellular domain of CD8ζ to the intracellular domain of CD3ζ has been shown to transduce signaling events typically associated with the TCR/CD3 complex when stimulated with the α-CD8 antibodies.5,46 Unlike the TCR α/β-chains, which are modified predominantly by N-linked glycans, the CD8ζ chimera contains O-linked glycans.35,33,57–59 This difference in glycan modification on an otherwise common signaling background allowed us to examine the ability of PTxB and a diverse panel of lectins to activate canonical CD3 signaling via receptors modified with N-linked or O-linked glycans. PTxB

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Figure 6. PTx induces calcium flux in CD8ζ Jurkat cells. WT, TCR−, or CD8ζ Jurkat cells were loaded with the Fluo-4 NW calcium indicator dye and stimulated with 1 μg/mL anti-CD3 (A), 1 μg/mL anti-CD8 (B), 5 μg/mL PTxB (C), or 5 μg/mL boiled PTxB (D). The time at which ligand is added is marked with a black arrow. Data were collected on a Molecular Dynamics FlexStation. Representative data are shown for each treatment.
has at least four glycan binding sites and can bind sialylated and nonsialylated N-glycans, sialylated O-glycans, and sialylated gangliosides.23 In previous studies,25−32 PTxB has been shown to activate a variety of receptors, including the TCR, TLR4, and CD14. In this study we have shown that PTxB also activates the CD8ζ chimera. Specifically, the CD8ζ chimera rescued PTxB-activated signaling events in TCR−Jurkat cells, including an increased level of tyrosine phosphorylation of TCR signaling molecules (e.g., ZAP-70), accumulation of inositol phosphates, activation of ERK, and mobilization of intracellular Ca2+ (Figures 3−6). Together, these results strongly support the hypothesis that PTxB does not recognize amino acid sequences present on receptor molecules but rather acts like a superlectin to promote signaling by clustering a wide variety of glycosylated receptors.

We did observe some differences in the magnitude of signaling when comparing TCR versus CD8ζ activity. While the anti-CD8 antibodies induced signaling via the CD8ζ chimera to levels comparable to that of anti-CD3 stimulation of the TCR (Figures 3−6), PTxB-mediated activation and lectin-mediated activation of signaling via these two receptors were not always equivalent. For example, PTxB activated ERK (Figure 5) and mobilized Ca2+ (Figure 6C) more efficiently on cells expressing wild-type TCR than on cells expressing the CD8ζ chimera, suggesting there is an inherent difference between the TCR and CD8ζ complexes that makes the TCR a better receptor for PTxB. Whether this is attributed to binding efficiency or some other factor remains unclear at this point.

We also examined the ability of various plant lectins to initiate activation of signaling activities downstream of the wild-type TCR or CD8ζ chimera, including mobilization of intracellular Ca2+ and activation of ERK (Figures 7−9 and Table 1). The activation patterns for many of these lectins, either via the TCR (containing N-linked glycans) or via the CD8ζ chimera (containing O-linked glycans), could be predicted on the basis of their preference for binding to N- or O-linked glycans. However, some of the lectins displayed activities in the biological assays that would not have been predicted on the basis of their glycan binding profiles in cell-free systems. For example, while PHA-E has not been previously reported to bind to O-linked glycans, it does activate signaling via the CD8ζ chimera (Table 1). The preferred ligand for PHA-E is particularly complex, requiring three different binding elements: a bisecting GlcNAc group, a Gal group at the nonreducing terminus of the Man(α1−6) branch, and a GlcNAc group on the Man(α1−3) branch.64 In this case, the preferred ligand represents the highest-affinity ligand discovered through careful biochemical analyses, and while each of the binding elements is required for high-affinity binding, some ligands lacking an individual element still bound, albeit with reduced affinity. A branched N-linked glycan can display different sugars on each branch and can satisfy this requirement for different adjacent glycans. In contrast, O-linked glycans display minimal branching, and a single glycan cannot provide the three binding elements for PHA-E. However, as seen in Figure 1B, each CD8 monomer possesses five closely spaced sites for O-linked modification. Activation by PHA-E could be mediated by the simultaneous engagement of closely spaced heterogeneous O-linked glycans. Thus, biochemical binding assays, such as glycan arrays using a single purified O-glycan, are unlikely to reveal the diversity that is allowed in biological systems such as the one described here.

Figure 7. Lectin-mediated Ca2+ signaling. WT, TCR−, or CD8ζ Jurkat cells were loaded with the Fluo-4 NW calcium indicator dye and stimulated with 2 μg/mL WGA (A), SBA (B), PHA-L (C), ConA (D), PNA (E), or DBA (F). The time at which ligand is added is marked with a black arrow. Data were collected on a Molecular Dynamics FlexStation. Representative data are shown for each treatment.

Figure 8. PHA-L utilizes the TCR but not CD8ζ to activate signaling events. WT or CD8ζ Jurkat cells were untreated or stimulated with 10 μg/mL PHA-L for 2 h, and inositol phosphates were analyzed (n = 2).
Another deviation from predicted results is the failure of PNA to activate the CD8ζ chimera. PNA is reported to recognize Galβ1−3GalNAc, also called Thomsen-Friedenreich antigen (T-antigen), which is a major O-glycan structure found on human T cells (Table S3 of the Supporting Information). However, the inability of PNA to mediate cellular aggregation suggests Jurkat cells lack PNA receptor expression. In contrast, another lectin, Jacalin, which also binds the T-antigen, can promote signaling (Table 1), suggesting that T-antigen is expressed on Jurkat cells. An explanation for these seemingly contradictory results is that binding of PNA but not Jacalin is inhibited when the T-antigen is further modified by sialylation, suggesting that the majority of T-antigen present on Jurkat cells is sialylated. This is further supported by the observation that tumor cells, like the Jurkat cell line, express an increased level of sialylation compared to that of normal cells.

It is also intriguing that some lectin−receptor complexes promoted either Ca2+ or ERK signaling, but not both (Table 1). PHA-L and DSL activated CD8ζ leading to ERK, but not Ca2+, signaling. In contrast, GSL II and LEL activated TCR leading to Ca2+, but not ERK, signaling. The ability to activate either ERK or Ca2+ but not both could be due to quantitative or qualitative differences in receptor activation, although it is difficult to imagine how quantitative differences could lead to weakened Ca2+ signaling in one case and weakened ERK signaling in another. Activation of the TCR promotes receptor translocation and clustering into signaling regions called immunological synapses or supramolecular activation clusters (SMACs). It is likely that the spatial orientation of the receptors within the SMAC can drastically affect the ability to support and sustain signaling activity. The complex and dynamic structure of the immunologic synapse makes it very difficult to study. Lectins capable of promoting the assembly of TCR- or CD8ζ-mediated signaling complexes with incomplete functional capacity (e.g., Ca2+ but not ERK) are very useful tools for studying the dynamic assembly of intermediates involved in the TCR signaling process.

Finally, we found that three lectins, ECL, VVA, and Jacalin, promoted ERK signaling, but not Ca2+ signaling, in cells lacking the TCR or the CD8ζ chimera (Table I and Figure 9). These results suggest that these lectins are capable of activating T cells by a TCR-independent signaling pathway, which has yet to be identified.

In summary, we have developed a model system for examining the ability of PTxB and other lectins to activate canonical CD3 signaling via either N-linked or O-linked glycans. Our results confirm the hypothesis that biological responses to lectins cannot be predicted simply on the basis of binding preferences determined from glycan array binding.

Figure 9. Calcium and ERK MAPK signaling induced by lectins ECL, VVA, and Jacalin. WT, TCR−, or CD8ζ Jurkat cells were loaded with the Fluo-4 NW calcium indicator dye and stimulated with 2 μg/mL ECL (A), VVA (B), or Jacalin (C). The time at which ligand is added is marked with a black arrow. Data were collected on a Molecular Dynamics FlexStation. WT (D) or TCR− (E) Jurkat T cells were left unstimulated or stimulated with the indicated lectin (2 μg/mL) for 15 min. Whole cell extracts were analyzed by Western blotting for activated ERK with phospho-specific antibodies. Representative data are shown for each treatment.
studies. Furthermore, the ability to cross-link a given receptor is not the sole prerequisite for driving signaling, because different lectins elicit quantitative and qualitative differences in downstream responses. This model system will be useful for probing the specific details involved in receptor activation, including defining the importance of microdomains and membrane substructures critical for recruitment of various signaling molecules to activated receptors.

ASSOCIATED CONTENT

Supporting Information
Glycosylation profiles for the TCR and CD8 and links to Center for Glycomics lectin–glycan binding results (Tables S1–S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS

ConA, concanavalin A; DBA, D. biflorus agglutinin; DSL, D. stramonium lectin; ECL, E. cristagalli lectin; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; GSL II, G. simplificofolia lectin II; ITAM, immunoreceptor tyrosine-based activation motif; JAC, Jacalin; LCA, L. culinaris agglutinin; LEL, L. esculentum lectin; Man, mannose; Neu5Ac, N-acetylneuraminic acid; PHA, phytohemagglutinin; PHA-L, phytohemagglutinin leucoagglutinin; PHA-E, phytohemagglutinin erythroagglutinin; PNA, peanut agglutinin; PSA, P. sativum agglutinin; PTx, pertussis toxin; PTxB, pertussis toxin B subunit; RCA, R. communis agglutinin; SBA, soybean agglutinin; SJA, S. japonica agglutinin; sWGA, succinylated wheat germ agglutinin; TCR, T cell receptor; UEA-I, U. europaeus agglutinin; VVA, V. villosa agglutinin; WGA, wheat germ agglutinin; WT, wild-type.

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